Sample Preparation and Identification Techniques for Chemical Warfare and Mid-Spectrum Agents

A General Survey for the NATO AC/225 LG/7-SIBCA Handbook, Chapter 8

J.R. Hancock, P.A. D'Agostino and L.R. Provost
Defence Research Establishment Suffield
Box 4000, Stn Main
Medicine Hat, Alberta T1A 8K6

"The use of this information is permitted subject to recognition of proprietary and patent rights."

DEFENCE RESEARCH ESTABLISHMENT SUFFIELD

Technical Report
DRES TR 1999-034

March 1999

Défense nationale National Defence

Canada

UNCLASSIFIED

DEFENCE RESEARCH ESTABLISHMENT SUFFIELD
RALSTON ALBERTA

TECHNICAL REPORT 1999-034

SAMPLE PREPARATION AND IDENTIFICATION TECHNIQUES FOR CHEMICAL WARFARE AND MID-SPECTRUM AGENTS

A GENERAL SURVEY FOR THE NATO AC/225 LG/7-SIBCA HANDBOOK, CHAPTER 8

by

J.R. Hancock, P.A. D'Agostino and L.R. Provost

ABSTRACT

Sample preparation and identification techniques for chemical warfare and mid-spectrum agents were surveyed as part of Canada's contribution to a joint NATO project to create a NATO Handbook on the sampling and identification of biological and chemical agents. Sample preparation techniques such as solid phase extraction, supercritical fluid extraction and derivatization were reviewed with respect to their applicability to chemical warfare and mid-spectrum agents. Identification techniques including; gas chromatography, liquid chromatography, mass spectrometry, Fourier transform infrared spectroscopy and nuclear magnetic resonance spectroscopy were examined in light of the need to confirm the identity of these agents in the presence of complex background matrices.

Executive Summary

Title: J.R. Hancock, P.A. D'Agostino and L.R. Provost, Sample Preparation and Identification Techniques for Chemical Warfare and Mid-Spectrum Agents:A General Survey for the NATO AC/225 LG/7-SIBCA Handbook, Chapter 8, Suffield Technical Report 1999-034, March 1999, UNCLASSIFIED.

Introduction: The Canadian Forces (CF) may be called on to conduct peacekeeping or peacemaking operations in regions of the world where there is a significant threat of chemical/biological warfare agent use. To operate effectively in these theatres the CF must be able to identify the exact nature of the chemical/biological agent(s). As part of NATO, Canada may be required to collect, package, transport and analyze samples believed to contain chemical warfare or mid-spectrum agents.

Results: Sample preparation and identification techniques for chemical warfare and mid-spectrum agents were surveyed as part of Canada's contribution to a joint NATO project to create a NATO Handbook on the sampling and identification of biological and chemical agents. Sample preparation techniques such as solid phase extraction, supercritical fluid extraction and derivatization were reviewed with respect to their applicability to chemical warfare and mid-spectrum agents. Identification techniques including; gas chromatography, liquid chromatography, mass spectrometry, Fourier transform infrared spectroscopy and nuclear magnetic resonance spectroscopy were examined in light of the need to confirm the identity of these agents in the presence of complex background matrices.

Significance of Results: The CF may be deployed as part of a NATO combined force in regions of the world where there is a significant threat of chemical/biological warfare agent use. Identification of CB agents is of importance since the results of such analyses would contribute to the development of strategic and political positions and would facilitate the dissemination of technical advice to in-theatre field commanders and medical personnel.

Future Goals: The CB threat spectrum includes chemical and biological warfare agents and toxins of biological origin in the "mid-spectrum" between these agents. The CF needs the ability to collect, transport and identify all agents in the threat spectrum. In addition the problems associated with radiological agents must be addressed. DRES will initiate an effort to integrate the disparate requirements for these agents into a single sample collection and transport system.

TABLE OF CONTENTS

1.0 Introduction

2.0 Chapter 8.0: Sample Preparation and Identification of CW and Mid-Spectrum Agents

- 8.1 Introduction
- 8.2 Sample Storage and Custody
- 8.3 Sample Preparation Techniques for CW Agents
  - 8.3.1 General Considerations
  - 8.3.2 Liquid/Liquid Extraction
  - 8.3.3 Solid Phase Extraction
  - 8.3.4 Liquid/Solid Extraction
  - 8.3.5 Supercritical Fluid Extraction
  - 8.3.6 Headspace
  - 8.3.7 Thermal Desorption
  - 8.3.8 Derivatization
  - 8.3.9 Solvent Evaporation/Concentration
- 8.4 Sample Preparation Techniques for Mid-Spectrum Agents
  - 8.4.1 General Considerations
  - 8.4.2 Particulate Removal
  - 8.4.3 Lyophilization
  - 8.4.4 Digestion
  - 8.4.5 Derivatization
- 8.5 Identification Techniques for CW and Mid-Spectrum Agents
  - 8.5.1 General Considerations
  - 8.5.2 Criteria for Identification of CW Agents
  - 8.5.3 Criteria for Identification of MS Agents
  - 8.5.4 Gas Chromatography
  - 8.5.5 High Performance Liquid Chromatography
  - 8.5.6 Capillary Electrophoresis
  - 8.5.7 Mass Spectrometry
  - 8.5.8 Infrared Spectroscopy
  - 8.5.9 Nuclear Magnetic Resonance Spectroscopy
- 8.6 Sample Preparation and Identification Schemes
  - 8.6.1 Chemical Warfare Agents
  - 8.6.2 Mid-Spectrum Agents (Peptide/Proteins)
- 8.7 Preparatory Work in Peacetime
- 8.8 Quality Assurance and Control

1.0 INTRODUCTION

NATO troops during peacekeeping or battlefield operations may be exposed to chemical or biological warfare agents. NATO, therefore, must posses the ability to collect, transport and analyze suspected contaminated samples in order that the agent(s) can be identified. Once the agent has been identified, NATO can properly respond to such an event. Real time detection, while possible for chemical warfare agents, is currently under development for biological agents. Nonetheless, unambiguouus identification of the agent(s) will require the use of instrumentation found in specialized national laboratories. This is especially true for the case of first use of chemical or biological agents or when conflicting results are obtained with field detectors (as may happen with the use of a novel chemical agent).

The NATO AEP-10 Handbook on Sampling and Identification of Chemical Agents (SICA) describes recommended procedures and techniques for the field sampling, packaging, transportation and identification of chemical warfare agents. The Handbook provides general guidance to personnel responsible for carrying out sampling and identification activities in support of NATO command and intelligence requirements. Under the guidance of the NATO Army Armaments Land Group 7 on NBC Defence, the SIBCA (Sampling and Identification of Biological and Chemical Agents) Subgroup undertook to produce a integrated Handbook which would deal with the operational and laboratory requirements for sampling and identification of biological and chemical agents. The SIBCA Handbook will update and replace the existing fourth edition of the AEP-10 Handbook and the NATO Handbook on the sampling and identification of biological agents.

National representatives to the SIBCA subgroup were tasked with preparing individual chapters for the Handbook. The Handbook will consist of the following nine chapters; Chapter One: Introduction, Chapter Two: Sampling Techniques, Chapter Three: Mobile Identification Systems, Chapter Four: Reporting, Chapter Five: Training, Chapter Six: Sample Preparation and Identification Techniques for BW agents, Chapter Seven: BW Databases, Chapter Eight: Sample Preparation and Identification Techniques for CW and Mid-Spectrum Agents and Chapter Nine: CW Databases. The handbook deals with operational issues such as sampling, transport, mobile identification and training. As well, the Handbook will deal with laboratory issues such as sample preparation and identification techniques and reference databases.

This report surveys information on sample preparation and identification techniques for chemical warfare and mid-spectrum agents and forms the basis of Canada's contribution to Chapter 8 of the SIBCA Handbook.

2.0 CHAPTER 8: SAMPLE PREPARATION AND IDENTIFICATION OF CW AND MID-SPECTRUM AGENTS

8.1 INTRODUCTION

All samples arriving at the laboratory must be assumed to contain either toxic or infectious material and the appropriate precautions taken in handling such samples. Analysis of these samples must be carried out at a properly equipped laboratory experienced in the identification of chemical warfare, mid-spectrum and biological warfare agents (see Annex A). It is unlikely that battlefield samples arriving at the laboratory will be in a form suitable for direct analysis. In most cases, sample preparation will be necessary prior to analysis.

8.2 SAMPLE STORAGE AND CUSTODY

The results of the analysis of samples suspected of being contaminated with chemical warfare, mid-spectrum or biological warfare agents may be used for military (battlefield), political (allegations of use) or verification (CWC or BWC) purposes. It is therefore mandatory that the handling and analysis of the samples be carried out with a view of maintaining a continuous chain of custody.

When the sample arrives at the laboratory, unless the contents of the package are known, it must be assumed that the sample contains a biological agent and the package must be stored in a Level 3 containment suite. The integrity of the sealed sample and packaging should be confirmed by visual inspection. In cases where samples are collected from the battlefield, comments on the integrity of the package or sample shall be noted by the analyst and reported with the analytical results. In political or verification cases where there are questions regarding either the integrity of the package or the sample, no analysis would be performed and the sample would be stored pending discussions with the originator. As a general practice, photographs of the exterior of the sample package and contents should be obtained for future reference.

The responsible scientist maintains written notes in which he/she assigns the sample a unique sample number and describes the handling of the sample. As the package is opened a real time chemical agent detector should be used to monitor the contents for chemical agent vapours and the responsible scientist shall date and initial each layer of packaging. The packaging material is retained during the analysis of the sample. Once the sample has been unwrapped, the sample number, date and initials of the responsible scientist are placed on the sample. As far as possible, a portion of the sample(s) should be retained for possible future examination.

The sample is then stored safely in a locked cabinet which is under the sole control of the scientist. If the sample is transferred to another scientist, a transfer form is completed which details the transfer. The second scientist now initials and dates the sample thus showing when it was placed into his/her custody. The detailed history of the sample(s) should be available to guide the analysts toward the most appropriate analytical method. When not in use the sample is returned to the locked cabinet.

8.3 SAMPLE PREPARATION TECHNIQUES FOR CW AGENTS

8.3.1 General Considerations

Samples, prior to analysis, may be subjected to a variety of preparation techniques. Depending on the type of sample and identification technique used, sample preparation can accomplish some or all of the following objectives:

(a) improvement in sensitivity through the removal of chemical interference(s) associated with the sample matrix;

(b) improvement in sensitivity through concentration and/or derivatization of the analyte;

(c) increase the analyte volatility by derivatization; and

(d) removal of sample components that can damage sensitive instrument hardware such as GC columns, HPLC pumps and columns or mass spectrometer sources.

Analysis of samples for the unambiguous identification of CW agents is similar to the analysis for scheduled chemicals under the Chemical Weapons Convention. Detailed recommended operating procedures for the analysis of these chemicals have been validated during a series of international round-robin exercises.

Selected References

1. Recommended Operating Procedures for Sampling and Analysis in the Verification of Chemical Disarmament, FIN, 1994.

2. AC/225 (LG/7-SIBCA) D/10 (6), Research Report on Verification of the Chemical Weapons Convention, Part XIII - Evaluation of Recommended Operating Procedures for Sampling and Analysis, NO, 1998, UNCLASSIFIED.

8.3.2 Liquid/Liquid Extraction (LLE)

Liquid/liquid extractions are primarily used for extracting analytes from an aqueous matrix into a water immiscible organic solvent. Extraction efficiency is a function of the distribution ratio of the analyte between the two phases and the ratio of the volumes of aqueous to organic solvent.

Liquid/liquid extractions can be used to quantitatively preconcentrate an analyte with concentration factors of about ten fold being typical. There are numerous water immiscible organic solvents; however, in practice only a few such as hexane and dichloromethane are routinely used for the LLE of chemical warfare agents. Emulsion formation, especially when using chlorinated solvents is a common problem. In practice, a known volume of the aqueous sample is combined with a smaller volume of organic solvent (ratios of 10:1 are common) and the two phases vigorously mixed, allowed to separate and the organic layer removed. Depending on the extraction efficiency, additional extractions with fresh solvent may be required. Finally the organic layers are combined, concentrated and analyzed.

LLE may result in the cleanup of a sample by selectively extracting the analyte of interest from a "dirty" matrix. Although a solvent may extract an analyte with high efficiency from an aqueous sample, it is likely that the solvent will have co-extracted a range of other compounds. For this reason, chromatographic separations will likely be required as part of the analysis.

Selected References

1. AC/225 (Panel VII/SICA) N/269, 1993 United Nations Round Robin Analytical Exercise: Canadian Report, CA, 1993, UNCLASSIFIED.

2.AC/225 (Panel VII/SICA) N/286, Analysis of Samples for the 4th Round Robin Exercise, NL, 1993, UNCLASSIFIED.

8.3.3 Solid Phase Extraction (SPE)

Solid phase extraction is a physical extraction technique in which an analyte, usually in aqueous solution, is passed through a solid adsorbent bed. Under proper conditions, the analyte interacts with the adsorbent and is selectively extracted from the aqueous solution. SPE is often used as an alternative to liquid/liquid extractions of aqueous samples.

Solid phase adsorbents are typically modified silica which are packed into cartridges ready for use. The adsorbent is solvated with an appropriate organic solvent and then washed (generally with water for aqueous samples) prior to the application of the sample. The sample is applied to the cartridge, and passed through using either vacuum or positive pressure. The retained analyte is

desorbed from the cartridge using a solvent of sufficient strength to interrupt the analyte/adsorbent interaction.

This form of extraction can be used to quantitatively preconcentrate an analyte with concentration factors of 10-100, or to cleanup a sample by retaining the analyte of interest during removal of matrix components. Typical solvents used in SPE include: hexane, dichloromethane, isopropanol and acetone. Emulsion formation is not a problem as is the case in liquid/liquid extraction. There are documented cases of the irreversible adsorption/ decomposition of chemical warfare agents on solid adsorbents; therefore, detailed studies need to be carried out with the analyte of interest prior to the use of solid adsorbents. As with liquid/liquid extraction, the extraction process is not totally specific. It should be expected that a range of other compounds from the sample will be retained and subsequently desorbed from the adsorbent.

Solid phase micro extraction (SPME) is a relatively new form of SPE. It combines sample preparation and GC-injection into one step. Analytes are adsorbed on a polymeric fiber coated with a stationary phase by placing the fiber into a water sample. Stationary phases used in SPME are apolar or moderately polar. Typical phases are: polyacrylate, poly(dimethylsiloxane), poly(dimethylsiloxane)/divinylbenzene and Carbowax/divinylbenzene. The fibers are thermally desorbed into the GC injection port at 250°C. This technique compliments existing methods and has been recently applied to the analysis of nerve agents in water.

Selected References

1. AC/225 (LG/7) D/4 (13), Determination of CWA in Natural Water Samples by Solid-Phase Microextraction, SWE, 1997, UNCLASSIFIED.

2. AC/225 (LG/7) D/10 (5), Collaborative Studies Between Poland and The Netherlands on the Analysis of Chemical Warfare Agents and Related Compounds Part 2: Solid Phase Microextraction, PO/NL, 1998, UNCLASSIFIED.

3. AC/225 (Panel VII/SICA) N/175, Sampling and Analysis of Sarin Isolated from Water, NL, 1990, UNCLASSIFIED.

4. AC/225 (Panel VII/SICA) N/201, Use of Sorbent Extraction in Verification of Alleged Use of CWA - Part 9, NO, 1991, UNCLASSIFIED.

5. AC/225 (Panel VII/SICA) N/211, A Solid Phase Extraction Method for the Verification of the Decomposition Products of Mustard in Aqueous Samples, CA, 1991, UNCLASSIFIED.

6. AC/225 (Panel VII/SICA) N/174, Determination of VX in Water by Thermospray LC-MS, NL, 1990, UNCLASSIFIED.

8.3.4 Liquid/Solid Extraction (LSE)

Liquid/solid extraction is a physical extraction technique used for extracting analytes from solid matrices such as soil, adsorbents or man-made materials. Organic solvents are typically used as the extracting solvent, although water has been used successfully in a number of cases. Due to the volumes of solvents employed, this form of extraction is typically used for sample cleanup rather than concentration.

Extraction is carried out by placing a sample of the solid material into a vessel containing an appropriate solvent. For short extraction times (up to 1/2 hour), the use of ultrasonification can enhance the extraction of organics from solids. For longer times, a soxhlet type extractor is often used. In general a suitable solvent must be chosen that will extract the analyte of interest from the matrix, but leave behind the bulk of the undesired matrix components. It is possible to carry out sequential extractions with various solvents or using water at different pH values in order to selectively remove analytes from the matrix. If the analyte is absorbed on a man-made material such as a paint, clothing or an aerosol filter, care must be taken in choosing a solvent which will not react with or dissolve the sample material.

Selected References

1. AC/225 (Panel VII/SICA) N/269, 1993 United Nations Round Robin Analytical Exercise: Canadian Report, CA, 1993, UNCLASSIFIED.

2.AC/225 (Panel VII/SICA) N/286, Analysis of Samples for the 4th Round Robin Exercise, NL, 1993, UNCLASSIFIED.

3.AC/225 (Panel VII/SICA) N/248, International Collaborative "Round Robin III" Test for Verification Analysis, GE, 1992, UNCLASSIFIED.

4.AC/225 (Panel VII/SICA) N/257, 1991 United Nations Round Robin Analytical Exercise: Canadian Report, CA, 1992, UNCLASSIFIED.

5.AC/225 (Panel VII/SICA) N/238, Preparation and Analysis of Samples for the Third Round Robin Verification Exercise, NL, 1992, UNCLASSIFIED.

8.3.5 Supercritical Fluid Extraction (SFE)

Supercritical fluid extraction is a physical extraction technique in which analytes are extracted from either solid or liquid matrices by a fluid maintained at supercritical conditions. Typically the matrix is a solid such as soil and the technique may be used to cleanup the sample or preconcentrate the analyte of interest.

SFE offers the potential of high concentration factors where the analyte is extracted from the matrix and deposited onto a media followed by elimination of the supercritical fluid.

In the laboratory, a sample is loaded into an extraction thimble and installed in the extractor. A supercritical fluid is then either allowed to flow through the thimble (dynamic extraction) or to fill the thimble for a preset time (static extraction). The density of the supercritical fluid and the temperature under which the extraction is carried out will influence the extraction efficiency. The supercritical fluid carrying the analyte is then depressurized, vented to atmosphere and the extracted analyte(s) recovered.

Although a relatively new technique, it is clear that no single SFE trapping method has proven to be suitable for all analytes. Trapping methods include; bubbling through a solvent, depressurizing the fluid into a vial (either empty or containing glass beads) and depressurizing the fluid onto a solid phase extraction cartridge. Although carbon dioxide is currently the most commonly used supercritical fluid, it does not possess a very high solvating power and for this reason modifiers such as methanol are added to the fluid.

Selected References

1. AC/225 (Panel VII/SICA) D/58 (9), Influence of Ageing on the Supercritical Fluid Extraction of Pollutants in Soils, FR, 1995, UNCLASSIFIED.

2. AC/225 (Panel VII/SICA) D/58 (10), Supercritical Fluid Extraction of an Organophosphorous Compound from Soils with Capillary GC Analysis, FR, 1995, UNCLASSIFIED.

8.3.6 Headspace

Headspace sampling can be used for the sample cleanup and concentration of analytes from the vapour above liquid or solid samples. Although there are a number of variations in how headspace sampling is carried out, typically the vapour above a sample is collected and analyzed for the presence of volatile compounds.

Concentration factors for these systems are difficult to calculate, but it is clear that preconcentration will occur when the analyte is trapped onto a solid adsorbent. In practice, it is useful to be aware that the sample collected for analysis is only representative of the analytes in the vapour phase. The varying volatilities of analytes will result in an inaccurate reflection of the composition of the bulk sample.

Selected References

1. AC/225 (Panel VII/SICA) N/159, Headspace Gas Chromatography - A New Technique in Verification of Alleged Use of CWA - Part 8, NO, 1990, UNCLASSIFIED.

8.3.7 Thermal Desorption

Thermal desorption is not normally considered a sample preparation technique. It is generally regarded as a means of sample introduction into an analytical instrument. However given the range of matrices (solid-gases) that are amenable to thermal desorption, the technique occupies a unique place among sample preparation techniques.

Thermal desorption when combined with a chromatographic technique can be used to analyze volatiles collected using the headspace technique described previously. In some cases, portions of a solid sample may be placed in the thermal desorption apparatus itself and desorbed directly. Care should be taken as thermal desorption may cause decomposition of the sample and the creation of artifacts.

For liquids or extracts in volatile organic solvents, relatively large volumes (e.g. > 100 µL) may be injected onto adsorbent tubes followed by removal of the solvent using a gentle stream of inert gas. The preconcentration of analytes arises due to differences in "breakthrough volumes" (adsorption affinities) between the solvent and the analytes.

Analysis of the samples is carried out by heating the tube which thermally desorbs the organics into a gas chromatograph. Proper temperature selection during the thermal desorption step may permit selective desorption of certain materials to the exclusion of others depending, in part, on the relative volatilities and adsorbent affinities exhibited by the adsorbed analytes.

Selected References

1. AC/225 (Panel VII/SICA) N/163, Selective Thermal Desorption GC Analysis of Compounds of Chemical Defence Interest, CA, 1990, UNCLASSIFIED.

2. AC/225 (Panel VII/SICA) N/173, Thermal Desorption GC Analysis of Adsorbent Packed Minitubes Containing VX, CA, 1990, UNCLASSIFIED.

8.3.8 Derivatization

Derivatization of analytes may be necessary in order to improve sensitivity and/or enhance volatility for GC analysis. This technique is most frequently employed during the analysis of samples containing the hydrolysis products of chemical warfare agents.

Some general derivatization methods applicable to chemical warfare agents and their hydrolysis products are described as follows:

(a) Diazoalkylation. Diazomethane is used to enhance the volatility of chemical warfare agent hydrolysis products. The principal disadvantage of this method is that diazomethane is a toxic, potentially explosive compound.

(b) Silylation. Silylation is used to enhance the volatility of chemical warfare agent hydrolysis products. Trimethylsilyl trifluoroacetamide (BSTFA) and N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide(MTBSTFA) are typically used to produce trimethylsilyl and t-butyl dimethylsilyl derivatives of chemical warfare agent hydrolysis products. T-butyldimethylsilyl derivatives have the advantage of being more stable in the presence of small amounts of water.

(c) Pentafluorobenzylation. Pentafluorobenzyl bromide is used to enhance the volatility of chemical warfare agent hydrolysis products and has the added advantage of improving sensitivity during GC with electron capture or negative-ion chemical ionization mass spectrometric detection. The derivatization reaction requires several hours and the use of sodium hydride and 18-crown-6 ether.

(d) Methylation. Trimethylphenylammonium hydroxide is used to methylate the hydrolysis products of chemical warfare agents. An advantage of this method is that trimethylphenylammonium hydroxide may be used, in one step, to elute and derivatize the hydrolysis product(s) trapped on an ion exchange column.

(e) Thioesterification. GC analysis of lewisite is hampered by either the thermal decomposition or adsorption of lewisite. Sensitivity is significantly enhanced by derivatizing Lewisite with either 3,4-dimercaptotoluene or 1,2-ethanediol. The disadvantage of this method is that both lewisite and its major hydrolysis

product, lewisite oxide, form the same derivative, making it impossible to determine whether the original sample contained the chemical warfare agent lewisite, the hydrolysis product or both.

In general, derivatization should be avoided unless absolutely necessary, as it alters the analyte and increases the opportunities for sample contamination and artifact formation.

Selected References

1. AC/225 (Panel VII/SICA) N/210, Preliminary Results from the GC Analysis of the 3,4 Dimercaptotoluene Derivative of Lewisite, CA/DA, 1991, UNCLASSIFIED.

2.AC/225 (Panel VII/SICA) N/93, GC/MS Studies of t-Butyldimethylsilyl Derivatives of some Alkyl Phosphonic and Alkyl Methylphosphonic Acids, CA, 1987, UNCLASSIFIED.

3.AC/225 (Panel VII/SICA) N/107, Mass Spectra of t-Butyldimethylsilyl Esters of Organophosphorus and Glycolic Acids, NL, 1987, UNCLASSIFIED.

8.3.9 Solvent Evaporation/Concentration

Many of the sample preparation techniques described above produce sample extracts in organic solvents requiring further concentration prior to instrumental analysis. Concentration by solvent evaporation can be achieved through the use of a Kuderna-Danish evaporator or by solvent blowdown using a gentle stream of nitrogen. The key considerations for concentration by solvent reduction are summarized below:

(a) highly volatile agents (e.g. GB) may be lost if taken to dryness;

(b) trace contaminants in the solvent will become concentrated and may interfere with the analysis. This problem may be minimized by using high purity solvents.

(c) some solvents or their stabilizers may react with chemical warfare agents leading to the formation of artifacts. For example, alcohols react readily with some chemical warfare agents and their use during sample preparation should be avoided.

8.4 SAMPLE PREPARATION TECHNIQUES FOR MID-SPECTRUM AGENTS

8.4.1 General considerations

Samples, prior to analysis, will be subjected to a variety of preparation techniques. Depending on the type of sample and identification technique used, sample preparation can accomplish some or all of the following objectives:

(a) improvement in sensitivity through the removal of chemical interference associated with the sample matrix;

(b) improvement in sensitivity through concentration of the analyte;

(c) increase the analyte volatility by derivatization; and

(d) removal of sample components that can damage sensitive instrument hardware such as GC columns, HPLC pumps and columns or mass spectrometer sources.

(e) aids in structural characterization of peptides and proteins (e.g. enzymatic digestion and derivatization).

8.4.2 Particulate Removal

In general, it is assumed that a sample containing a mid-spectrum agent will either be collected as an aqueous sample (from a high volume aerosol sampler) or dissolved in water prior to further sample handling or analysis by electrospray mass spectrometry (ESI-MS). The removal of particulates from an aqueous samples is necessary prior to analysis in order to minimize damage to analytical instrumentation. Particulate removal may be accomplished by filtration or centrifugation.

With filtration, an aqueous sample is passed through a filter using positive pressure or vacuum. The filter itself can be composed of a variety of materials such as stainless steel, cellulose acetate, teflon, nylon and polyvinylidene difluoride. Depending on the volume to be filtered, the filter unit may attach directly to a low volume syringe or may be part of a larger filtration apparatus. Low recovery of peptides or proteins due to adsorption onto the filter is a potential problem especially with stainless steel and cellulose acetate filters.

Ultrafiltration is a technique for separating molecules in a solution based on their effective size. This filter is a selective semipermeable membrane that retains most macro-molecules above a certain size while permitting smaller molecules to pass into the filtrate. These filters are

characterised with a molecular weight cut off but do not retain all the molecules larger than the nominal pore size because some molecules deform and pass through the filter. It has been reported that ultrafiltration may lead to significant losses of proteinaceous compounds.

Centrifugation is a separation technique in which the rotation of a sample at high speeds (e.g. 2-3000 g) generates a centrifugal force which can be used to remove particulates.

Selected References

1. AC/225 (Panel VII/SICA) D/58 (5), Identification of Ile-Ser-Bradykinin in Aqueous Samples by Liquid Chromatography with Ultra Violet and Electrospray Mass Spectrometric Detection, CA, 1995, UNCLASSIFIED.

8.4.3 Lyophilization

Lyophilization or freeze drying is a technique for removing moisture from a sample by freezing the sample and subsequently subliming away the moisture. The three basic steps in this technique are: 1) freezing, 2) sublimation and 3) dehydration. This technique is widely used for the preservation of biomolecules. For example, peptides or proteins, containing methionine are unstable in aqueous solutions as they are oxidised to the corresponding sulphoxide. For this reason, peptides or proteins are typically purchased and stored as lyophilised powders.

8.4.4 Digestion

Digestion is a chemical or enzymatic process in which the chemical bonds between the amino acids of the proteinaceous mid-spectrum agents are cleaved resulting in a series of smaller peptides. The most frequently employed enzymatic digestion process involves the use of trypsin. Trypsin selectively cleaves the peptide linkage on the C terminal side of lysine and arginine. The resultant lower mass peptide fragments may be characterized on the basis of molecular mass by mass spectrometry. These masses constitute a mass map, that may be used to aid in the identification of the mid-spectrum agent.

Proteinaceous mid-spectrum agents or their enzymatic fragments may also be subjected to Edmans degradation, a chemical process that results in the sequential cleavage of the peptide linkages. This process uses phenylisothiocyanate which reacts with the amino acid on the N terminal of the peptide. Each amino acid adduct that is cleaved from the peptide is identified by HPLC-UV. Successful application of Edmans degradation will result in the acquisition of the complete amino acid sequence for the mid-spectrum agent or its enzymatic fragment

Selected References

1. AC/225 (Panel VII/SICA) D/60 (10), Determination of Staphylococcal Enterotoxin B by On-Line (Micro) Liquid Chromatography Electrospray Mass Spectrometry, NL, 1996, UNCLASSIFIED.

2. AC/225 (LG/7-SIBCA) D/4 (3), Sample Handling and Analysis Protocol for Toxic and Bioactive Peptides: A Conceptual Approach, CA/NO, 1997, UNCLASSIFIED.

3. AC/225 (LG/7-SIBCA) D/4 (7), The First International NATO/SICA Training Exercise on the Identification of Unknown Peptides, NL, 1997, UNCLASSIFIED.

4. AC/225 (LG/7-SIBCA) D/10 (15), A Method for the Sample Handling and Analysis of Bio-Active Peptides, CA/NO, 1998, UNCLASSIFIED.

5. AC/225 (LG/7-SIBCA) D/10 (10), Characterization of Cholera Toxin by Liquid Chromatography-Electrospray Mass Spectrometry, NL, 1998, UNCLASSIFIED.

8.4.5 Derivatization

Chemical derivatization of a proteinaceous mid-spectrum agent may be used to characterize the amino acids in the agent. The presence of disulfide bridges can be determined using dithiothreitol which cleaves the disulfide bond between two cysteine amino acids. The number of basic amino acids can be determined by acetylation using acetic anhydride which will increase the molecular mass by 63 Da for each basic amino acid. The number of acidic amino acids can be determined by methylation which will result in an increase in molecular mass of 14 Da for each acidic amino acid.

Selected References

1. AC/225 (LG/7-SIBCA) D/10 (15), A Method for the Sample Handling and Analysis of Bio-Active Peptides, CA/NO, 1998, UNCLASSIFIED.

2. AC/225 (LG/7-SIBCA) D/10 (10), Characterization of Cholera Toxin by Liquid Chromatography-Electrospray Mass Spectrometry, NL, 1998, UNCLASSIFIED.

8.5 IDENTIFICATION TECHNIQUES FOR CW AND MID-SPECTRUM AGENTS

8.5.1 General Considerations

Analysis of samples suspected of containing chemical warfare or mid-spectrum agents, especially when the agent is present in a complex matrix or when the agent is new or unusual must be carried out at a properly equipped laboratory experienced in the identification and confirmation of these agents.

Samples should be taken and transported to an identification laboratory according to the procedures described in Chapter 2 of this Handbook. Samples should be sent to at least two laboratories experienced in the analysis of CW agents.

Currently, three analytical techniques can provide the structural information which satisfies the criteria for unambiguous identification of chemical warfare and mid-spectrum agents given in Sections 8.5.2 and 8.5.3. These techniques are; mass spectrometry, infrared (IR) spectrometry and nuclear magnetic resonance (NMR) spectrometry.

Gas chromatography-mass spectrometry is currently the technique of choice for CW agent separation, detection and identification. Liquid chromatography-mass spectrometry (LC-MS) with electrospray ionization is currently the technique of choice for mid-spectrum agents separation, detection and identification.

8.5.2 Identification Criteria for Chemical Warfare Agents

Three levels of identification with increasing levels of certainty have been defined.

PROVISIONAL IDENTIFICATION: A chemical warfare agent may be considered provisionally identified when one of the following criteria has been met:

I The chromatographic retention data acquired for the chemical warfare agent measured under two different experimental conditions matches that of a known chemical warfare agent; or

II The chromatographic retention data acquired for the chemical warfare agent with a specific detector (FPD, TID, AED) matches that of a known chemical warfare agent; or

CONFIRMED IDENTIFICATION: The identification of a chemical warfare agent is confirmed when one of the following criteria has been met:

I A complete spectrum acquired using a single spectrometric technique (MS, NMR or IR) matches the corresponding reference spectra in a database. If the molecular ion is not present in the mass spectrum, techniques such as chemical ionization must be carried out to confirm the molecular mass of the compound

II The chromatographic retention data acquired for the chemical warfare agent during mass spectrometric analysis using selected ion monitoring (minimum of three ions) matches that of a known chemical warfare agent. The ratio of the three ions must fall within 10% of the values of an authentic reference standard. The ions should have coincident maxima, the same peak width at half height and exhibit a signal to noise ratio greater than three.

UNAMBIGUOUS IDENTIFICATION: Unambiguous identification provides the highest level of certainty required for the development of strategic positions. The identification of a chemical warfare agent is unambiguous when the following criterion has been met:

I The chromatographic retention data acquired for the chemical warfare agent and spectra acquired using two different spectrometric techniques (MS, NMR or IR) match those obtained for an authentic reference standard under identical experimental conditions. If the molecular ion is not present in the mass spectrum, techniques such as chemical ionization must be carried out to confirm the molecular mass of the compound.

8.5.3 Identification Criteria for Mid-Spectrum Agents

Three levels of identification have been defined as follows to indicate the increasing level of certainty associated with each.

PROVISIONAL IDENTIFICATION: A mid-spectrum agent may be considered provisionally identified when one of the following criteria has been met:

I The chromatographic retention data acquired for the mid-spectrum agent under two different experimental conditions matches that of known mid-spectrum data; or

II The molecular mass of the mid-spectrum agent, determined by MS, matches that of known mid-spectrum agent data; or

III A specific immunological assay registers a positive response.

CONFIRMED IDENTIFICATION: The identification of a mid-spectrum agent is confirmed when any two of the three criteria for provisional identification are met or:

I In the case of proteinaceous mid-spectrum agents, the molecular mass and corresponding mass map of the enzymatic digestion products (with a minimum of three products) matches that of known mid-spectrum agent data.

UNAMBIGUOUS IDENTIFICATION: Unambiguous identification provides the highest level of certainty required for the development of strategic and political positions. The identification of a mid-spectrum agent is unambiguous when the following conditions are met:

For non-proteinaceous mid-spectrum Agents

I The chromatographic retention data acquired for the mid-spectrum agent and spectra acquired using two different spectrometric techniques (MS, NMR or IR) match those for authentic reference standards acquired under identical experimental conditions. If the molecular ion is not present in the mass spectrum, techniques such as chemical ionization or electrospray mass spectrometry must be carried out to confirm the molecular mass.

For proteinaceous mid-spectrum agents:

I The chromatographic retention data acquired for the mid-spectrum agent under two different experimental conditions matches that of an authentic reference standard acquired under identical experimental conditions or a specific immunological assay registers a positive response; and

II The molecular mass and corresponding mass map of the enzymatic digestion products (with a minimum of three products) matches that for an authentic reference standard acquired under identical experimental conditions; and

III Sequence data for the mid-spectrum agent matches that for an authentic reference standard acquired under identical experimental conditions.

8.5.4 Gas Chromatography

Gas chromatography (GC) is a technique, suited to the separation of gases or volatile compounds. In gas chromatography, a multicomponent sample, in the gas phase, is resolved into its various components. The first step in this technique is the introduction of the sample into a chromatographic column where the separation will occur. Liquid samples can be injected into the

column using either an on-column injector or a split/splitless injector. Gas samples are injected using: gas sampling valves, headspace injection or thermal desorption.

The majority of analyses are carried out under temperature programming conditions, where the column temperature is increased with time. As the sample moves through the column it partitions between the carrier gas and a stationary liquid phase. Each component of the sample spends a different length of time in the liquid phase. The time that it takes for a component to travel through the column is related to the time spent in the liquid phase and is known as its retention time. The retention time for a component on a specific column is a reproducible characteristic of the component.

In practice, retention time data are difficult to reproduce between different laboratories due to variations in operating conditions and instrumental parameters. These difficulties may be overcome by using one of a variety of retention index systems that relate the retention time of the analyte to the retention times of a series of standards. In the Kovats system, the retention time of an analyte is related to that of a series of n-alkanes, while other systems make use of phosphorous and sulphur containing standards. Because it is possible for more than one compound to elute with the same retention time, retention indices for a given analyte should be determined on several columns, each of different stationary phase polarity (e.g. DB-1, DB-5, DB1701), in order to increase the certainty of the analyte identity.

Detection of the components as they elute from the chromatographic column may be performed by a variety of universal and selective detectors. The most common is the flame ionization detector which responds to the majority of organic compounds. When dealing with complex matrices, selective detectors such as flame photometric (FPD, which responds to sulphur or phosphorous), thermionic or nitrogen phosphorous (TID, which responds to nitrogen or phosphorous) and atomic emission (AED, which responds to the presence C, N, P, F, S, As, Cl and Br) detectors may provide element specific information. Although selective detectors provide information on the presence or absence of certain elements they do not provide structural information. Mass spectrometers and infrared spectrometers provide this type of information and for this reason are widely interfaced to chromatographic techniques.

Selected References

1. AC/225 (Panel VII/SICA) D/56 (13), Capillary Gas Chromatographic Analysis of Nerve Agents Using Large Volume Injections, NL, 1994, UNCLASSIFIED.

2. AC/225 (Panel VII/SICA) D/56 (14), Gas Chromatographic Retention Index Database of Compounds of Chemical Defence Interest, NL, 1994, RESTRICTED.

3. AC/225 (Panel VII/SICA) N/159, Headspace Gas Chromatography - A New Technique in Verification of Alleged Use of CWA - Part 8, NO, 1990, UNCLASSIFIED.

4. AC/225 (Panel VII/SICA) N/205, Canadian Contribution to SICA Retention Index Data Base of Compounds of Chemical Defence Interest, CA, 1991, UNCLASSIFIED.

5. AC/225 (Panel VII/SICA) N/215, Analysis of CW Agents by Means of Large Volume Injections Into a Capillary GC Column, NL, 1991, UNCLASSIFIED.

6. AC/225 (Panel VII/SICA) N/254, Large Volume Injections for Enhanced Detection Limits During GC-FTIR Analysis of Chemical Warfare Agents, CA, 1992, UNCLASSIFIED.

7. AC/225 (Panel VII/SICA) N/34, Analysis of Mycotoxins in Blood by Derivatisation Procedures, US, 1984, UNCLASSIFIED.

8. AC/225 (Panel VII/SICA) N/36, Some Experiences with Analysis of Trichothecene Mycotoxins by Electron Capture/GC, UK, 1984, UNCLASSIFIED.

9. AC/225 (Panel VII/SICA) N/110, Decomposition of Trifluoroacetyl Derivatives on Fused Silica Capillary Columns during Gas Chromatographic Analysis, NL, 1987, UNCLASSIFIED.

8.5.5 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is a separation technique in which a multicomponent sample, in the liquid phase, is resolved into its various components. HPLC is well suited to the separation of thermally labile or low volatility compounds that cannot be easily analyzed by GC. The first step in this technique is the introduction of the sample into a chromatographic column where the separation occurs. Sample introduction in HPLC is accomplished with a switching valve equipped with a fixed volume sample loop. The sample is injected as a liquid plug into the flow stream and swept onto the HPLC column for separation.

Separations can be performed under isocratic or gradient elution conditions with reverse phase (e.g. C_8 or C_{18} columns) separations being the most common. Separations are based on the physical interactions of the sample components with the liquid mobile phase and a stationary phase. Under reverse phase gradient elution conditions, the composition of the mobile phase increases in organic content over time. The time that it takes for a component to travel through the column is known as the retention time and for a specific column is a reproducible characteristic of each component.

Detection in HPLC is typically performed with an ultraviolet/visible detector. Nerve, blister and mid-spectrum agents do not generally possess strong chromophores, therefore sensitivity and

selectivity remains poor for these compounds. In recent years flame photometric and thermionic detectors have been used for selective identification with microcolumn liquid chromatography. Although selective detectors provide information on the presence or absence of certain elements they do not provide structural information. Mass spectrometers provide this type of information and for this reason are widely interfaced to chromatographic techniques.

Selected References

1. AC/225 (Panel VII/SICA) N/197, Application of Chemiluminescence for the Detection of Thiodiglycol in Aqueous Matrices Using High Performance Liquid Chromatography, US, 1990, UNCLASSIFIED.

2. AC/225 (Panel VII/SICA) N/236, Determination of Organophosphorous Compounds by Micro-column LC with On-Line Flame Photometric and Thermionic Detection, NL, 1992, UNCLASSIFIED.

3. AC/225 (Panel VII/SICA) N/250, Detection of Thiodiglycol and Its Sulfoxide and Sulfone Analogues in Environmental Waters by High Performance Liquid Chromatography, US, 1992, UNCLASSIFIED.

4. AC/225 (Panel VII/SICA) N/277, Large-Volume Injections in Microcolumn-LC Using Flame Photometric Detection, NL, 1993, UNCLASSIFIED.

5. AC/225 (Panel VII/SICA) N/278, Micro-LC Analysis of the Enatiomers of VX with Thermionic Detection, NL, 1993, UNCLASSIFIED.

6. AC/225 (Panel VII/SICA)D/56 (3), Microbore HPLC Separation of Substance P and Related Peptides, CA, 1994 UNCLASSIFIED.

7. AC/225 (Panel VII/SICA) N/37, The Analysis of Some Trichothecene Mycotoxins by Liquid Chromatography, UK, 1984, UNCLASSIFIED.

8.5.6 Capillary Electrophoresis (CE)

Capillary electrophoresis employs narrow bore (10-200 mm i.d., 370 mm o.d. polyimide coated) capillaries to perform high efficiency separations of both large and small molecules based on their mobilities in an electric field. The instrument consists of two buffer reservoirs connected via a hollow capillary column, a high voltage power supply, and a detection system.

Samples are introduced onto the CE column either electrokinetically (an applied potential) or hydrodynamically (pressure on the sample reservoir forces the sample into the capillary). A

potential is then applied across the capillary and the analytes are induced to move (migrate) in this applied field. Because of differences in the effective mobilities (resulting in different migration velocities) of different substances in an electric field, the mixture separates into discrete zones of individual substances. The analytes are detected on-column by ultraviolet (UV) or laser induced fluorescence (LIF) through a small window on the capillary. As was the case with HPLC, CE has been interfaced with mass spectrometry in order to provide molecular mass and structural information.

Different modes of capillary electrophoresis can be performed using a standard CE instrument. These include capillary zone electrophoresis, capillary gel electrophoresis, micellar electrokinetic capillary chromatography, capillary isoelectric focusing and capillary isotachophoresis.

Capillary zone electrophoresis (CZE) is the simplest and most widely used technique in CE. The capillaries used for CZE are bare fused silica containing surface silanol groups. The separation mechanism is mainly based on differences in solute size and charge at a given pH.

As opposed to CZE, capillary gel electrophoresis (CGE) separates species on the differences in solute size as the analytes migrate through the pores of a gel-filled capillary allowing for separation based on "molecular sieving". They also minimise solute diffusion and prevent solute adsorption to the capillary walls.

Micellar electrokinetic capillary chromatography (MEKC) involves a micellar solution of an ionic surfactant to provide a phase for a chromatographic separation and allow for the separation of neutral species.

Isoelectric focusing is a separation method in which analytes are separated on the basis of their isoelectric points or pI values. Capillary isotachophoresis uses a discontinuous buffer system between which sample zones form as the analytes concentrate between the front and end constituents. Where, as in CZE the electropherogram gives distinct peaks like those found in HPLC, in capillary isotachophoresis (CITP) the isotachopherogram gives a series of steps each representing an analyte zone.

Selected References

1. AC/225 (Panel VII/SICA) D/58 (3), Capillary Zone Electrophoresis Analysis of Bio-Active Peptides, CA, 1995, UNCLASSIFIED.

2. AC/225 (Panel VII/SICA)D/56 (10), Capillary Electrophoresis Coupled On-Line with Flame Photometric Detection, NL, 1994 UNCLASSIFIED.

3. AC/225 (Panel VII/SICA)D/60 (7), Capillary Electrophoresis Analysis of Chemical Warfare Agent Breakdowns Part 1: Counterelectroosomotic Separation of Alkylphosphonic Acids and Their Monoester Derivatives, FR, 1996, UNCLASSIFIED.

4. AC/225 (Panel VII/SICA) D/60 (15), Use of Capillary Electrophoresis for the Detection of Chemical Warfare Agent Breakdown Products, US, 1996, UNCLASSIFIED.

8.5.7 Mass Spectrometry

Mass spectrometry (MS) is an identification technique in which sample molecules are ionized, the ionized molecules and any lower mass fragmentation ions that may have been formed are then separated on the basis of their mass to charge ratio. Assignment of the structure of the original molecule is done by either fundamental interpretation of the data or by comparing the acquired data to reference data. In most cases chromatographic separation proceeds detection as it allows the individual components of a mixture to be introduced into the MS as relatively pure components. Mass separation can be accomplished using quadrupole, sector (electrostatic and magnetic), time of flight, ion trap or Fourier Transform instruments. High resolution measurements are possible with sector or Fourier Transform instruments. This data can be used determine elemental composition.

The most widely used method of ionization for the GC-MS analysis of chemical warfare agents is electron impact (EI) ionization. High energy electrons produced within the mass spectrometer source interact with sample molecules, resulting in the loss of an electron by the molecule. The resulting molecular ion may undergo fragmentation leading to the formation of lower mass fragmentation ions. In some cases the molecular ion, used to determine molecular mass, is not observed. Chemical ionization (CI), a much less energetic ionization technique, has proven very useful for providing complementary molecular ion information. Methane, isobutane and ammonia reagent have all been used as CI gases during the analysis of chemical warfare agents.

In tandem mass spectrometry multiple analysers (e.g. sector or quadrupole) are combined into a single instrument. Four common modes of operation are: product ion scan, precursor scan, constant neutral loss and reaction ion monitoring. The combination of two or more analysers may result in the extraction of more structural information and/or greater selectivity than may be achieved with a single analyser.

Thermospray mass spectrometry and more recently atmospheric pressure ionization (e.g. electrospray, ionspray and atmospheric pressure CI) techniques have enabled the direct mass spectrometric analysis of chemical warfare agent hydrolysis products in aqueous samples. Both techniques may be interfaced to HPLC or CE for component separation, with thermospray have been largely superceeded by atmospheric pressure ionization (API) for most applications. API-MS, a relatively gentle ionization technique, generally produces molecular ion information in either the

positive or negative ion mode. Under positive ion mode, molecular adducts, dimers and trimers are often observed. Under appropriate API conditions, it is possible to promote collisionally activated dissociation in the interface resulting in the formation of product ions that may aid in structural identification.

Electrospray ionization (ESI) is the most widely used ionization technique for the LC-MS analysis of mid-spectrum agents. ESI-MS generally produces multiply charged molecular ions of the general form, $(M+nH)^{n+}$. These ions may be used to establish the molecular mass of the mid-spectrum agent. Under appropriate ESI conditions, it is possible to promote collisionally activated dissociation in the interface resulting in the formation of product ions that may aid in structural identification. Amino acid sequence information for peptides and peptide fragments may be determined under these conditions. Tandem mass spectrometry can also be used for this purpose.

Matrix assisted laser desorption ionization (MALDI) represents a complimentary means of ionization for mid-spectrum agents. Analyses are generally performed without chromatographic separation, a disadvantage in the case of multicomponent samples. The principal advantage of this technique is it's ability to ionize mid-spectrum agents with molecular mass in excess of 100 kDa.

Selected References

1. AC/225 (Panel VII/SICA) N/235, The Determination of Mustard Gas and Related Vesicants in Rubber and Paint, NL, 1992, UNCLASSIFIED.

2. AC/225 (Panel VII/SICA) N/255, Capillary Column GC-MS/MS Analysis of Chemical Warfare Agents: Acquisition of Daughter Spectra, CA, 1992, UNCLASSIFIED.

3. AC/225 (Panel VII/SICA) N/285, Mass Spectral Data of Alkyl methylphosphonofluoridates, NL, 1993, UNCLASSIFIED.

4. AC/225 (LG-7-SIBCA) D/4 (2), The First Contribution of the Czech Republic to the SICA Database, CZR, 1997, UNCLASSIFIED.

5. AC/225 (LG/7-SIBCA) D/10 (7), Analytical Data of Some O-alkyl-N,N-Diakylphosphoramidocyanidates, CZR, 1998, UNCLASSIFIED.

6. AC/225 (LG/7-SIBCA) D/4 (11), Mass Spectra of Analytical Derivatives of Some CWA Degradation Products, PO, 1997, UNCLASSIFIED.

7. AC/225 (LG/7-SIBCA) D/10 (8), Packed Capillary Liquid Chromatography Electrospray Mass Spectrometry and Tandem Mass Spectrometry of Hydrolyzed HT and HQ, CA, 1998, UNCLASSIFIED.

8. AC/225 (LG/7-SIBCA) D/4 (14), The Use of LC/MS Techniques for the Verification of Precursors and Degradation Products of Nerve Agents, SWI, 1997, UNCLASSIFIED.

9. AC/225 (LG/7-SIBCA) D/4 (17), Identification of Isomeric Phosphonates and Isomeric Phosophonothioates by Tandem Mass Spectrometry, US, 1997, UNCLASSIFIED.

10. AC/225 (Panel VII/SICA) D/58 (4), High Resolution Electrospray Mass Spectrometry with a Magnetic Sector Instrument: Accurate Mass Measurement and Peptide Sequencing, CA, 1995, UNCLASSIFIED.

11. AC/225 (Panel VII/SICA) D/60 (10), Determination of Staphylococcal Enterotoxin B (SEB) by On-Line (Micro) Liquid Chromatography Electrospray Mass Spectrometry, NL, 1996, UNCLASSIFIED.

12. AC/225 (Panel VII/SICA) D/60 (1), Identification of Bioactive Peptides by High Resolution Liquid Chromatography-Electrospray Mass Spectrometry, CA, 1996, UNCLASSIFIED.

13. AC/225 (Panel VII/SICA) D/60 (2), Liquid Chromatography Mass Spectrometric and Tandem Mass Spectrometric Identification of Peptides Using Electrospray Ionization, CA, 1996, UNCLASSIFIED.

14. AC/225 (Panel VII/SICA) D/60 (11), Analysis of Peptide Mid-Spectrum Agents by Electrospray Mass Spectrometry, NL, 1996, UNCLASSIFIED.

15. AC/225 (LG/7-SIBCA) D/10 (10), Characterization of Cholera Toxin by Liquid Chromatography-Electrospray Mass Spectrometry, NL, 1998, UNCLASSIFIED.

8.5.8 Infrared Spectrometry

Infrared spectrometry (IR) is an identification technique in which sample molecules absorb radiation (typically in the 4000 to 400 cm^{-1} range. Pure samples may be introduced into the IR spectrometer either as a pellet (typically in a KBr matrix) or as a thin film between NaCl or KBr plates. In most cases, chromatographic separation proceeds detection as it allows the individual components of a mixture to be introduced into the IR as relatively pure components. Currently two different approaches are used to acquire the infrared spectrum of a component as it elutes from the chromatographic column. The first technique uses a direct deposition mechanism whereby the column effluent is deposited onto a moving ZnSe plate at extremely low temperatures. Condensed phase spectra are collected by either scanning the plate on the fly or in a post chromatographic run mode. The second technique, uses a gold plated light pipe through which the column effluent flows. Vapour phase IR spectra are acquired in real time, with higher detection limits than the direct deposition technique. Unlike MS, IR spectra are more difficult to interpret from first principles and

identification of a chemical warfare agent is based on the comparison of the acquired spectrum to that obtained for a reference standard.

Selected References

1. AC/225 (Panel VII/SICA) N/176, The Identification of Lewisite Residues at the Former Chemical Weapons Storage Site at Bowes Moor, UK, 1990, UNCLASSIFIED.

2. AC/225 (Panel VII/SICA) N/247, Performance of a Gas Chromatography/Infrared Spectrometry System, GE, 1992, UNCLASSIFIED.

3. AC/225 (Panel VII/SICA) N/256, DRES Vapour Phase Infrared Spectral Database of Compounds of Chemical Defence Interest: 1992 Update, CA, 1992, UNCLASSIFIED.

4. AC/225 (Panel VII/SICA) N/262, Infrared Spectra of Schedule 1 Compounds. O-alkyl methylphosphonofluoridates, GE, 1993, UNCLASSIFIED.

5. AC/225 (LG/7-SIBCA) D/10 (3), Sampling and Analysis Procedures Used in the Investigation of a Cylinder Suspected of Containing an NBC Agent, CA, 1998, UNCLASSIFIED.

6. AC/225 (Panel VII/SICA) D/60 (8), Propyl-Mustard From World War II Stocks, GE, 1996, UNCLASSIFIED

8.5.9 Nuclear Magnetic Resonance Spectrometry

Nuclear magnetic resonance spectrometry (NMR) is an identification technique in which sample molecules absorb radio frequency radiation (typically in the 100 to 400 MHz range for protons) and the resulting absorbance spectrum is used to uniquely identify the original molecule. Assignment of the structure of the chemical warfare agent is done by either fundamental interpretation or by comparison of the acquired spectrum to that obtained for a reference standard.

In NMR spectrometry, the sample is analyzed as a dilute solution. Deuterated solvents such as: D_2O, d_6-DMSO and $CDCl_3$ are used to prepare these solutions with TMS frequently being used as the internal reference. Because NMR does not generally use a separation technique prior to detection (such as GC in MS and FTIR), samples must be in a relatively pure form to avoid spectral interferences.

Instrumentation equipped with multi-nuclear capability (especially ^{13}C, ^{31}P, ^{19}F and ^{1}H) can be useful for identifying chemical agents but detection levels vary depending on the nuclei selected. Using a phosphorous probe and an acquisition time of 8 hours, 10 ppm of a nerve agent can be

detected with a modern superconducting NMR. With a proton probe under similar conditions the detection limits would be approximately 1 ppm. The use of selective probes such as ^{31}P and ^{19}F provide for selective detection of CW agents in the presence of a complex background. Special NMR techniques or experiments such as multi-dimensional NMR, indirect detection or special probe designs can also increase specificity in complex backgrounds. Where sufficient sample is present, the information obtained by NMR can permit complete structural elucidation of new or previously unreported agents. Two dimensional NMR experiments such as homo- and heteronuclear correlation spectroscopy (COSY, HETCOR, NOESY, ROESY) are now standard methods that aid in the determination of structure.

Selected References

1. AC/225 (Panel VII/SICA) N/267, Carbon-13 Nuclear Magnetic Resonance Analysis of Pyridinium Oxime Therapeutic Agents, CA, 1993, UNCLASSIFIED.

8.6 Sample Preparation and Identification Schemes

8.6.1 Chemical Warfare Agents

In general, the sample preparation and analysis of samples suspected of being contaminated with chemical warfare agents initially focuses on the identification of nerve agents and vesicants. If these compounds are not detected in the sample more extensive sample preparation and analysis techniques are employed. The following schemes for chemical warfare agents are examples of the steps that could be used for the specific target compounds in solid and liquid samples.

Solid Sample

Target Compounds: GA, GB, GD, GF, H, L and VX

Step 1: If the sample is not homogenous, homogenize gross sample. Where the amount of sample permits, subdivide gross sample into four or more subsamples and select one subsample for analysis.

Step 2: If the presence of compounds in the vapour above the solid sample is of interest, consider headspace sampling with analysis by gas chromatography, mass spectrometry or Fourier transform infrared spectroscopy.

Step 3: On the subsample, perform solid liquid extraction using one of the following techniques; sonification, soxhlet extraction or supercritical fluid extraction. The following example will use sonification to demonstrate a possible approach for extraction of a soil sample.

Add one milliliter of deuterated chloroform per gram of soil and sonicate for 10 minutes. As nerve agents may react with alcohols, only chloroform that is **not** stabilized with an alcohol can be used. Remove particulates by either filtering through a 0.45 µm filter or by centrifuging for 10 minutes at 2000 g. Transfer supernatant to clean sample vial. The extract can be then analyzed by gas chromatography, mass spectrometry, nuclear magnetic resonance or Fourier transform infrared spectroscopy. If necessary, the extract can be concentrated using solvent evaporation or solid phase extraction and re-analyzed.

Step 4: To check for the presence of lewisite by gas chromatography, the extract is derivativized using 3,4 dimercaptotoluene. Add 100µL of the extract to 100µL of 3,4-dimercaptotoluene (1mg/mL in chloroform) and let stand for one minute at 20°C. Analyze the derivative by either gas chromatography-mass spectrometry or gas

chromatography-Fourier transform infrared spectroscopy. If necessary, the extract can be concentrated using; solvent evaporation or solid phase extraction and re-analyzed.

Solid Sample

Target Compounds: Phosphonic Acids and Thiodiglycol

Step 1: If the sample is not homogenous, homogenize gross sample. Where the amount of sample permits, subdivide gross sample into four or more subsamples and select one subsample for analysis.

Step 2: On the subsample, perform solid liquid extraction using one of the following techniques; sonification, soxhlet extraction or supercritical fluid extraction. The following example will use sonification to demonstrate a possible approach for extraction of a soil sample.

Add one milliliter of water per gram of soil and sonicate for 10 minutes. Remove particulates by either filtering through a 0.45 µm filter or by centrifuging for 10 minutes at 2000 g. Transfer supernatant to clean sample vial. The extract can be then analyzed by liquid chromatography-mass spectrometry. If necessary, the extract can be concentrated using solvent evaporation and re-analyzed. In cases where NMR analysis is warranted, it will be necessary to evaporate the sample to dryness and reconstitute it in deuterated water.

Step 3: Alternatively, the water extract can passed through an ion exchange cartridge and then evaporated to dryness. To form the trimethyl sily derivatives of the phosphonic acids and thiodiglycol, one hundred microliters of dichloromethane, 100µL of BSTFA and 100 µL of pyridine are added to the dried sample. The solution is heated to 60°C for 90 minutes. The derivatives can then be analyzed by gas chromatography, mass spectrometry or Fourier transform infrared spectroscopy.

Liquid Sample (Aqueous)

Target Compounds: Phosphonic Acids and Thiodiglycol

Step 1: Where the amount of sample permits, subdivide gross sample into four or more subsamples and select one subsample for analysis. If necessary, remove particulates from the sample by either filtering through a 0.45 µm filter or by centrifuging for 10 minutes at 2000 g. Transfer supernatant to clean sample vial.

Step 2: The subsample can be analyzed directly by liquid chromatography-mass spectrometry. If necessary, the subsample can be concentrated using solvent evaporation and re-analyzed. In cases where NMR analysis is warranted, it will be necessary to evaporate the sample to dryness and reconstitute it in deuterated water.

Step 3: Alternatively, the water sample can passed through an ion exchange cartridge and then evaporated to dryness. To form the trimethyl sily derivatives of the phosphonic acids and thiodiglycol, one hundred microliters of dichloromethane, 100µL of BSTFA and 100 µL of pyridine are added to the dried sample. The solution is heated to 60°C for 90 minutes. The derivatives can then be analyzed by gas chromatography, mass spectrometry or Fourier transform infrared spectroscopy.

8.6.2 Mid-Spectrum Agents (Peptide/Protein)

In general, it is assumed that a sample containing a proteinaceous mid-spectrum agent will either be collected as an aqueous sample (from a high volume aerosol sampler) or dissolved in water prior to further sample handling or analysis by ESI-MS.

Step 1: Removal of Particulates/Adjust pH

The removal of particulates from an aqueous sample is necessary prior to analysis in order to minimize damage to analytical instrumentation. Centrifugation appears to be the best approach for the removal of particulates, since low recovery of Ile-Ser-Bradykinin, a bio-active peptide, has been reported even when using low binding filtration membranes.

The pH of the sample is measured and adjusted if necessary, at this step as subsequent sample handling steps, such as reductive alkylation or enzymatic digestion, require the pH of the sample be compatible with the reaction.

Step 2: Purity and Molecular Mass Determination

Rapid analysis using isocratic LC-ESI-MS conditions with a wide mass range scan under low resolution and low sampling cone voltage conditions may be used to determine the relative purity of the sample and monoisotopic or average molecular mass(es) of the major sample component(s). A wide mass range scan (e.g. 150-1500 Da) is used to ensure that most components from singly charged dipeptides to multiply charged polypeptides are detected. Molecular masses determined under low resolution (e.g. 2000, 10% valley definition) are sufficiently accurate for obtaining matches for target compounds in peptide databases. Low sampling cone voltages (e.g. 50 V) are used to minimize collisionally activated dissociation in the interface.

For low molecular mass compounds (<1500 Da), it may be possible to obtain amino acid sequence information by promoting collisionally activated dissociation (CAD) in the ESI interface. Increasing sampling cone voltages generally results in increased fragmentation leading to the formation of product ions that may be used for amino acid sequencing.

Step 3: Separation and Fraction Collection

In cases where the sample contains multiple components, it is recommended that the components be separated by HPLC and the fractions collected prior to subsequent sample handling steps.

Step 4: Determining the Presence of Disulfide Bonds

The three dimensional structure of proteins, while critical for biological activity, may restrict access of reagents thereby reducing the effectiveness of digestion and derivatization techniques. One technique for denaturing peptides (i.e. cause the peptide to unfold) is immersion in a boiling water bath. To ensure that the peptide does not re-assume it's original three dimensional shape upon cooling, it is immediately immersed in an ice bath, after removal from the boiling water bath.

Disulfide bridges between cysteine residues exist either between cysteines in the peptide chain (intramolecular) or between cysteines in different chains (intermolecular). These bridges may be broken using reductive alkylation, which may be carried out during peptide denaturing. Sample handling steps such as reductive alkylation require a pH 8. Ammonium bicarbonate buffer, a volatile buffer compatible with ESI-MS, can be used to adjust the pH.

Following reductive alkylation, the reaction product(s) may be analyzed by ESI-MS. When iodoacetamide is used as the alkylating reagent, the mass increases by 58.03 Da for each cysteine that reacts with the iodoacetamide. The molecular mass of the intact peptide should be compared to that of the reductively alkylated peptide(s). If the molecular mass(es) are greater than that of the intact peptide, this is indicative of intramolecular disulfide bond(s). If the molecular mass calculated from the reductively alkylated peptide is less than the intact peptide this is indicative of intermolecular disulfide bond(s).

Step 5: Enzymatic Digestion and Mass Mapping of Enzymatic Fragments

Enzymatic digestions can be performed either in solution or in a flow-through system. While digestions performed in solution may take 12 or more hours, equivalent flow-through systems digest peptides in a matter of minutes. A flow-through system may be assembled from a column

packed with trypsin immobilized on a support, a solvent delivery system, an injector and a detector to monitor the elution of the digestion products.

Most enzymes have a pH range within which they will optimally digest peptides. For example, trypsin works best at a pH close to 8 and adjustment of the sample pH is performed by adding a known volume of ammonium bicarbonate buffer solution.

The collection of lower mass peptide fragments may be characterized on the basis of molecular mass by mass spectrometry. These masses constitute a mass map, that may be used to aid in the identification of the mid-spectrum agent that was subjected to enzymatic digestion. This mass map should correspond to the majority of those predicted for the digestion of the peptide. Difficulties arise when there is incomplete cleavages during the digestion.

Step 6: Accessing Amino Acid Primary Sequence Data

The enzymatic digestion products would generally be analyzed by LC-ESI-MS under collisional activated disassociation (CAD) conditions that promote the formation of product ions that provide amino acid sequence data for the tryptic fragments. CAD may be carried out either in the ESI interface, or alternatively, in a collisional cell between the first and second mass analyzer. The amount and quality of the CAD data acquired will depend on the amount of sample, the mass of the analyte and its amino acid sequence.

8.7 PREPARATORY WORK IN PEACETIME

The availability of modern analytical techniques and methods is a prerequisite for the rapid identification of chemical warfare and mid-spectrum agents. However, the presence of instrumentation and methods does not by itself ensure the unambiguous identification of these agents, their precursors or decomposition products. Successful identification is dependent on the analysis being carried out by a team experienced in the handling, storage, preparation and analysis of CW and mid-spectrum agents. Therefore, periodically an analyst team familiar with the identification techniques should participate in interlaboratory testing, international round robin exercises and internal quality assurance testing to evaluate their capabilities.

Laboratories require a synthetic capability in order to provide authentic reference standards which can be utilized for unambiguous identification. The spectra acquired during analysis of these reference standards may be compiled into a computer searchable database. These databases may be used to match the acquired spectra to that obtained for reference standards.

8.8 QUALITY ASSURANCE AND CONTROL

Increasingly, analytical laboratories are being required to provide documented evidence that the results they produce meet a prescribed standard. Terms such as; good laboratory practice (GLP), quality assurance (QA), quality control (QC) and standard operating procedures (SOPs) are used to describe these standards. It remains a national responsibility to ensure that laboratories conform to national and if appropriate international standards. In short, it is necessary to demonstrate that accurate records are maintained by a laboratory, in order that experimental results can be verified by tracing the information back to the raw data. In addition maintenance and calibration records are also necessary for the wide variety of instrumentation used in the laboratory.

Standard operating procedures (SOPs) apply to both analytical methods and instrument maintenance. Using an analytical method as an illustration, a SOP would contain setup and evaluation procedures for the instrumentation prior to running a sample. It would also contain step by step instructions for sample preparation. Instrumental conditions would be detailed and examples of system response would be included as well as the type of quality control samples and the frequency at which they are to be analyzed. A SOP would also contain information on maintaining statistical run charts and troubleshooting instructions in cases where the system is out of statistical control or fails to operate correctly.

DOCUMENT CONTROL DATA

(Security classification of title, body of abstract and indexing annotation must be entered when the overall document is classified)

1. ORIGINATOR (the name and address of the organization preparing the document. Organizations for whom the document was prepared, e.g. Establishment sponsoring a contractor's report, or tasking agency, are entered in section 8.)

Defence Research Establishment Suffield
Box 4000, Stn Main
Medicine Hat, AB T1A 8K6 Canada

2. SECURITY CLASSIFICATION (overall security classification of the document, including special warning terms if applicable)

Unclassified

3. TITLE (the complete document title as indicated on the title page. Its classification should be indicated by the appropriate abbreviation (S,C,R or U) in parentheses after the title.)

Sample Preparation and Identification Techniques for Chemical Warfare and Mid-Spectrum Agents. A General Survey for the NATO AC/225 LG/7-SIBCA Handbook, Chapter 8 (U)

4. AUTHORS (Last name, first name, middle initial. If military, show rank, e.g. Doe, Maj. John E.)

Hancock, James R., D'Agostino, Paul A. and Provost, Lionel R.

5. DATE OF PUBLICATION (month and year of publication of document)	6a. NO. OF PAGES (total containing information. Include Annexes, Appendices, etc.)	6b. NO. OF REFS (total cited in document)
March 1999	31	71

6. DESCRIPTIVE NOTES (the category of the document, e.g. technical report, technical note or memorandum. If appropriate, enter the type of report, e.g. interim, progress, summary, annual or final. Give the inclusive dates when a specific reporting period is covered.)

Suffield Report

8. SPONSORING ACTIVITY (the name of the department project office or laboratory sponsoring the research and development. Include the address.)

DRES

9a. PROJECT OR GRANT NO. (if appropriate, the applicable research and development project or grant number under which the document was written. Please specify whether project or grant)	9b. CONTRACT NO. (if appropriate, the applicable number under which the document was written)
6ED13	

10a. ORIGINATOR'S DOCUMENT NUMBER (the official document number by which the document is identified by the originating activity. This number must be unique to this document.)	10b. OTHER DOCUMENT NOS. (Any other numbers which may be assigned this document either by the originator or by the sponsor)
DRES TR 1999-034	

11. DOCUMENT AVAILABILITY (any limitations on further dissemination of the document, other than those imposed by security classification)

(X) Unlimited distribution
( ) Distribution limited to defence departments and defence contractors; further distribution only as approved
( ) Distribution limited to defence departments and Canadian defence contractors; further distribution only as approved
( ) Distribution limited to government departments and agencies; further distribution only as approved
( ) Distribution limited to defence departments; further distribution only as approved
( ) Other (please specify):

12. DOCUMENT ANNOUNCEMENT (any limitation to the bibliographic announcement of this document. This will normally correspond to the Document Availability (11). However, where further distribution (beyond the audience specified in 11) is possible, a wider announcement audience may be selected.)

3. ABSTRACT (a brief and factual summary of the document. It may also appear elsewhere in the body of the document itself. It is highly desirable that the abstract of classified documents be unclassified. Each paragraph of the abstract shall begin with an indication of the security classification of the information in the paragraph (unless the document itself is unclassified) represented as (S), (C), (R), or (U). It is not necessary to include here abstracts in both offical languages unless the text is bilingual).

Sample preparation and identification techniques for chemical warfare and mid-spectrum agents were surveyed as part of Canada's contribution to a joint NATO project to create a NATO Handbook on the sampling and identification of biological and chemical agents. Sample preparation techniques such as solid phase extraction, supercritical fluid extraction and derivatization were reviewed with respect to their applicability to chemical warfare and mid-spectrum agents. Identification techniques including; gas chromatography, liquid chromatography, mass spectrometry, Fourier transform infrared spectroscopy and nuclear magnetic resonance spectroscopy were examined in light of the need to confirm the identity of these agents in the presence of complex background matrices.

4. KEYWORDS, DESCRIPTORS or IDENTIFIERS (technically meaningful terms or short phrases that characterize a document and could be helpful in cataloguing the document. They should be selected so that no security classification is required. Identifiers, such as equipment model designation, trade name, military project code name, geographic location may also be included. If possible keywords should be selected from a published thesaurus. e.g. Thesaurus of Engineering and Scientific Terms (TEST) and that thesaurus-identified. If it is not possible to select indexing terms which are Unclassified, the classification of each should be indicated as with the title.)

Sample Preparation

Identification

Chemical Warfare Agents

Mid-Spectrum Agents

NATO

SIBCA